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The Interaction of Reduced Nicotinamide–Adenine Dinucleotide Phosphate with Reduced Nicotinamide–Adenine Dinucleotide–Ubiquinone Reductase from Bovine Heart Mitochondria

By C. IAN RAGAN
Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

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Reduction of the chromophores of mitochondrial NADH–ubiquinone reductase by NADPH reaches only 50% of the extent of reduction by NADH, monitored at 450nm. This effect is due to autoxidation of an enzyme component at a higher rate than its reduction by NADPH.

Besides catalysing nicotinamide nucleotide transhydrogenase activity, submitochondrial particles and isolated Complex I (NADH–ubiquinone reductase) (Hatefi *et al.*, 1962) are also capable of oxidizing NADPH in the absence of NAD^+ (Hatefi & Hanstein, 1973). Although the rate of oxidation of NADPH by NADH dehydrogenase is extremely low compared with the rate of NADH oxidation (Hatefi & Hanstein, 1973), this enzyme is in such kinetic excess compared with the rest of the respiratory chain that NADPH oxidase activity catalysed by submitochondrial particles is quite significant compared with NADH oxidase activity, and, moreover, is a phosphorylating pathway (Hatefi & Hanstein, 1973). It is of some interest therefore to establish whether the pathway of NADPH oxidation is identical with that of NADH oxidation. Hatefi and associates (Hatefi & Hanstein, 1973; Djavadi-Ohaniance & Hatefi, 1975) have proposed that the pathways are not identical, on the basis of the following: (*a*) NADPH causes a smaller extinction decrease in the 450nm region of the spectrum of Complex I than does NADH; (*b*) NADPH cannot reduce e.p.r. (electron-paramagnetic-resonance) centre 1 of Complex I; (*c*) NADH oxidase activity and NADPH oxidase activity of submitochondrial particles are inactivated at different rates by trypsin. In this report, these results are re-examined and shown to be consistent with the alternative suggestion (Rydström *et al.*, 1973; Ragan *et al.*, 1974) that NADH and NADPH are oxidized via the same pathway but at widely different rates.

Materials and Methods

Complex I was prepared by the method of Hatefi *et al.* (1962). D-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and L-lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Dual-wavelength spectroscopic measurements were performed with a Perkin–Elmer dual-wavelength spectrophotometer. NADH- and NADPH–$K_3Fe(CN)_6$ reductase activities were measured at 420nm in 1ml final volume containing 0.67M-sucrose, 50mM-Tris/HCl, pH7.0 at 22°C, 0.1% Triton X-100, 1mM-$K_3Fe(CN)_6$, 0.1mM-NADH or 0.1mM-NADPH and enzyme. NADH oxidase was measured at 340nm in 1ml final volume containing sucrose/Tris buffer as above, 0.1% Triton X-100, 0.1mM-NADH and enzyme. Glucose oxidase (EC 1.1.3.4) was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Protein was determined by the method of Lowry *et al.* (1951).

Results and Discussion

In Fig. 1, the bleaching of Complex I by NADPH and NADH is compared. Only a partial reduction by NADPH was observed compared with that by NADH (Fig. 1*a*), which could be increased to the maximal extent (i.e. that obtained with NADH) by subsequent addition of NAD^+ (Fig. 1*b*). The second phase of the reduction was due to NADH, produced by transhydrogenation from NADPH. These results are very similar to those of Hatefi & Hanstein (1973). The alternative explanation that partial reduction by NADPH is a result of the very low rate of oxidation was tested by restricting the rate of entry of reducing equivalents from NADH, to see if the results obtained with NADPH could be reproduced. Fig. 1 also shows the effect of reducing Complex I with an excess of DL-3-hydroxybutyrate in the presence of NAD^+ and limiting amounts of D-3-hydroxybutyrate dehydrogenase. Fig. 1(*c*) shows that full reduction of Complex I could be obtained by this system, despite the somewhat higher mid-point potential of the DL-3-hydroxybutyrate/acetoacetate couple compared with the NADH/NAD^+ couple. In fact, since DL-3-hydroxybutyrate was present at such a high con-

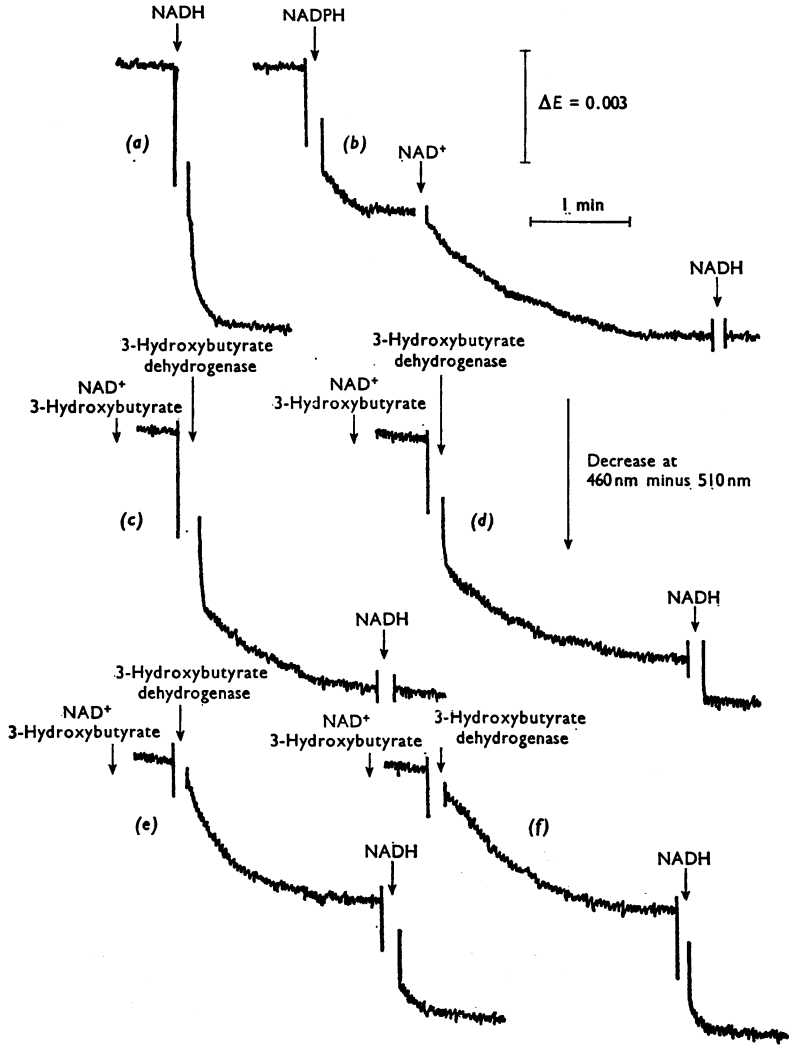

Fig. 1. *Reduction of Complex I by NADH and NADPH*

In each experiment, Complex I (0.5mg) was diluted to 1ml with 0.67M-sucrose, 50mM-Tris/HCl, pH7, 0.1% Triton X-100 (at room temperature) in a cuvette of 1cm light-path. Additions as indicated were NADH (0.1mM), NADPH (0.1mM), NAD^+ (0.1mM), DL-3-hydroxybutyrate (5mM) and D-3-hydroxybutyrate dehydrogenase: (*c*) 50μg; (*d*) 5μg; (*e*) 1.5μg; (*f*) 0.5μg.

centration (5mM), the effective reducing potential was probably much closer to that obtained with NADH (0.1mM) than the mid-point potentials would suggest. On decreasing the concentration of D-3-hydroxybutyrate dehydrogenase, the reduction became biphasic and incomplete (Fig. 1*d*), and eventually only partial reduction of Complex I was obtained (Figs. 1*e* and 1*f*). The extent of reduction in Figs. 1(*e*) and 1(*f*) was identical with that obtained with NADPH (Fig. 1*b*). Moreover, it is clear that NADPH was a more effective reductant than the system with 1.5μg of D-3-hydroxybutyrate dehydrogenase, but less effective (i.e. slower and less complete reduction) than the system with 5μg of D-3-hydroxybutyrate dehydrogenase.

To confirm that the extent of reduction of Complex I depended only on the rates of input of reducing equivalents, these were determined by measuring the rates in Complex I of the reduction of ferricyanide by NADH, NADPH and DL-3-hydroxybutyrate

Table 1. *Rates of input of reducing equivalents to Complex I*

NADH- and NADPH-$K_3Fe(CN)_6$ reductase activities were measured as described in the Materials and Methods section. Rates with D-3-hydroxybutyrate dehydrogenase were measured by the rate of reduction of $K_3Fe(CN)_6$ in the presence of excess of Complex I (5μg of protein). Results are presented as the input rates for 0.5mg of Complex I in 1ml (conditions of Fig. 1).

Substrate	D-3-Hydroxybutyrate dehydrogenase (μg of protein)	Input rate (nequiv./min)
NADH	—	53800
NADPH	—	9.2
NADPH + NAD^+	—	18.0
DL-3-Hydroxybutyrate + NAD^+	0.5	1.4
DL-3-Hydroxybutyrate + NAD^+	1.5	4.2
DL-3-Hydroxybutyrate + NAD^+	5.0	14.0
DL-3-Hydroxybutyrate + NAD^+	50	140

with various amounts of D-3-hydroxybutyrate dehydrogenase. These are presented in Table 1. The input rates are calculated for the amount of Complex I present in each of the experiments of Fig. 1. In agreement with the previous conclusions, the rates of input from NADPH lay between those obtained with 1.5μg and 5μg of D-3-hydroxybutyrate dehydrogenase. Moreover, in the presence of NADPH and NAD^+, the rate of input exceeded that obtained with 5μg of D-3-hydroxybutyrate dehydrogenase, explaining the full reduction of Complex I under these circumstances (Fig. 1*c*).

After reduction of Complex I by NADH as in Fig. 1(*a*), but in the presence of 20μg of L-lactate dehydrogenase, addition of pyruvate caused only partial reoxidation, indicating the presence of an NADH-reducible species of relatively high mid-point potential (probably greater than −200mV). After reduction by NADPH in the presence of L-lactate dehydrogenase, addition of pyruvate and NAD^+ caused no change in the extent of reduction, and further addition of NADH caused only a transient further reduction. These experiments show that the NADPH-reducible species is of high potential, whereas the low-potential component, i.e. that reoxidizable by pyruvate and NAD^+, is not reduced by NADPH. Therefore at low rates of input of reducing equivalents from NADH or NADPH the high-potential component(s) of Complex I is reduced, whereas reduction of the low-potential pool requires entry rates in excess of 28nequiv./min per mg of Complex I (from Table 1). The rate of oxidation of NADH by Complex I in the absence of any electron acceptor other than oxygen was found to be 30nequiv./min per mg of Complex I protein. Moreover, in an experiment similar to those of Fig. 1 the extent of bleaching of Complex I by NADPH was increased from 50% of that obtained with NADH to 91%, by inclusion of 10mM-D-glucose and 7×10^{-8}kat of glucose oxidase to maintain conditions close to anaerobic.

Thus the above findings may be explained by autoxidation of a low-potential component of Complex I. The explanation proposed probably accounts for the reduction of e.p.r. centre 2 (high-potential) by NADPH, but not e.p.r. centre 1 (low-potential) (Hatefi & Hanstein, 1973).

Although Djavadi-Ohaniance & Hatefi (1975) reported that the NADPH oxidase activity of submitochondrial particles was inactivated by trypsin (EC 3.4.21.4), and NADH oxidase was not, the NADH- and NADPH-$K_3Fe(CN)_6$ reductase activities of Complex I are inactivated at equal rates (Ragan, 1976). Since NADPH interaction with the dehydrogenase is rate-limiting for NADPH oxidase (Hatefi & Hanstein, 1973), whereas the interaction of the dehydrogenase with the rest of the respiratory chain is rate-limiting for NADH oxidase (Gutman & Singer, 1970), trypsin inactivation of the dehydrogenase would inhibit NADPH oxidase, but would have no effect on NADH oxidase until the residual dehydrogenase became rate-limiting.

In conclusion therefore the evidence that NADPH is oxidized by a distinct pathway can be adequately explained on the basis of the low rate of oxidation of NADPH by NADH dehydrogenase, without the need to postulate an alternative NADPH-dehydrogenation system.

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